
Product Manual

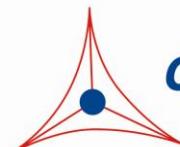
RAPAd® CMV Adenoviral Bicistronic Expression System (GFP)

Catalog Number

VPK-254

1 kit

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



CELL BIOLABS, INC.

Creating Solutions for Life Science Research

Introduction

Recombinant adenoviruses have tremendous potential in both research and therapeutic applications. There are numerous advantages they provide when introducing genetic material into host cells. The permissive host cell range is very wide. The virus has been used to infect many mammalian cell types (both replicative and non-replicative) for high expression of the recombinant protein. Recombinant adenoviruses are especially useful for gene transfer and protein expression in cell lines that have low transfection efficiency with liposome. After entering cells, the virus remains epichromosomal (i.e. does not integrate into the host chromosome so does not activate or inactivate host genes). Recently, recombinant adenoviruses have been used to deliver RNAi into cells.

Two methods have traditionally been used to generate recombinant adenoviruses. The first involves homologous recombination of a shuttle vector containing gene of interest and an adenoviral backbone plasmid vector (restricted in E1/E3) in an adenovirus packaging cell line. The isolation of recombinant adenovirus by this method involves performing multiple plaque isolations to avoid wild-type virus and is extremely laborious and time consuming. The second method, pAdEasy system, employs the homologous recombination machinery in *E. coli*, a recombinant adenovirus is produced by a double-recombination event between cotransformed adenoviral backbone plasmid vector and a shuttle vector carrying the gene of interest. For the pAdEasy method, the system is high fidelity, but inefficient and requires the screening of many bacterial colonies. This results in a significant time commitment even before transfection of recombinant DNA into E1-expressing cells such as HEK293 cells.

Cell Biolabs' RAPAd® Adenoviral Expression System provides a much faster and safer method to generate RCA-free recombinant adenovirus at high titer (see Table 1). The RAPAd® system uses a novel Ad backbone devoid of the left-hand ITR, the packaging signal and E1 sequences. There is no need to perform the bacterial *in vitro* homologous recombination (pAdEasy method), and also the multiple plaque isolations (standard homologous recombination method in packaging cell line). The RAPAd® system allows for generation of a recombinant virus within 2 weeks and the virus produced contained virtually no contaminating E1a sequences or replication-competent virus (RCA).

Cell Biolabs' RAPAd® Adenoviral Expression System is simple to use. The method is straightforward and requires very limited 'hands on' time from shuttle/backbone cotransfection to the isolation of virus particles. It produces equivalent infectious titers as the standard viral genome/shuttle plasmid recombination method.

In Cell Biolabs' RAPAd® CMV Adenoviral Bicistronic Expression System (GFP), the shuttle vector contains a CMV ahead of the multiple cloning sites followed by IRES-GFP.

Standard Homologous Recombination	pAdEasy Expression System	RAPAd® Expression System
Cotransfect 293 cells with Shuttle Vector and Ad Backbone Vector	Linearize Shuttle Vector using PmeI	Linearize Shuttle Vector and RAPAd® Ad Backbone Vector using PacI
↓ Multiple Plaque Isolations	↓ Cotransform <i>E.coli</i> BJ5183 cells with linearized Shuttle Vector and pAdEasy Vector	↓ Cotransfect 293 cells
↓ Virus Amplification	↓ Recombinant selection by restriction enzyme analysis	↓ Viral Stock
↓ Viral Stock	↓ Linearize recombinant plasmid using PacI	
	↓ Transfect 293 cells	
	↓ Viral Stock	
12-18 weeks	8-9 weeks	2-3 weeks

Table 1. Outline of Recombinant Adenovirus Systems

Related Products

1. AD-100: 293AD Cell Line
2. AD-200: ViraDuctin™ Adenovirus Transduction Reagent
3. VPK-090: ViraBind™ Lentivirus Concentration and Purification Kit
4. VPK-099: ViraBind™ Adenovirus Miniprep Kit
5. VPK-100: ViraBind™ Adenovirus Purification Kit
6. VPK-109: QuickTiter™ Adenovirus Titer Immunoassay Kit
7. VPK-110: QuickTiter™ Adenovirus Titer ELISA Kit
8. VPK-111: Rapid RCA Assay Kit
9. VPK-130: ViraBind™ Retrovirus Concentration and Purification Kit
10. VPK-250: RAPAd® Universal Adenoviral Expression System
11. VPK-251: RAPAd® RSV Adenoviral Expression System
12. VPK-253: RAPAd® miRNA Adenoviral Expression System

Kit Components

1. pacAd5 CMV-IRES-GFP Shuttle Vector (Part No. 325401): One 40 µL vial at 0.25 mg/mL.
2. pacAd5 9.2-100 Vector (Part No. 325002): One 40 µL vial at 0.25 mg/mL.
3. pacAd5 CMV-GFP Control Vector (Part No. 325004): One 40 µL vial at 0.25 mg/mL.
4. pacAd5 CMV-ntLacZ Control Vector (Part No. 325202): One 40 µL vial at 0.25 mg/mL.

Materials Not Supplied

1. 293 cells: we recommend 293AD Cell Line (Cat.# AD-100) for high titer production of recombinant adenovirus.
2. 293 Cell Culture Medium
3. Transfection Reagents
4. PacI (New England Biolabs, Cat.# R0547L)

Storage

Upon receipt, store all kit components at -20°C until their expiration dates.

Safety Considerations

Remember that you will be working with samples containing infectious virus. Follow the recommended NIH guidelines for all materials containing BSL-2 organisms.

Vector Features

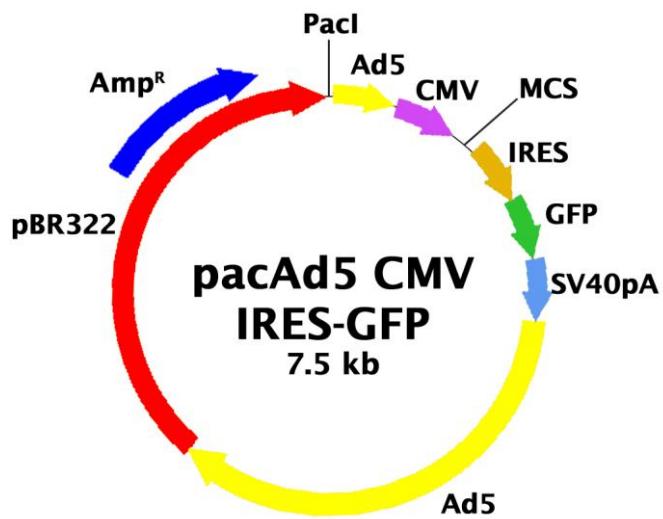


Figure 1. pacAd5 CMV-IRES-GFP shuttle Vector (7539 bp, Ampicillin-resistant).

pacAd5 CMV-IRES-GFP Features:

3-10:	PacI
16-368:	1-353 of Ad5
382-912	CMV Promoter
919-964:	MCS
983-1596:	IRES
1597-2316:	GFP
2317-2764:	SV40 pA
2759-5223:	3328-5792 of Ad5
6471-7331:	β Lactamase

Multiple Cloning Sites:

Pme I EcoR I
 TTGGTACCGTTAAACTCGAGGTCGACGGTATCGATAAGCTTGAATTCCTGCAGCCCCGGGGATCCACTAGT
Cla I EcoR V BamH I

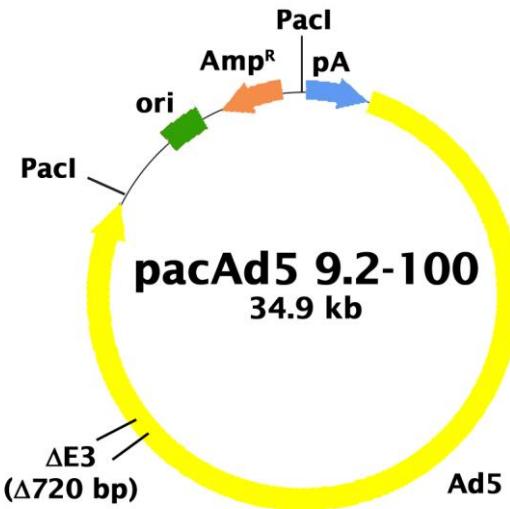


Figure 2. pacAd5 9.2-100 Vector (34947 bp, Ampicillin-resistant). The novel pacAd5 9.2-100 Ad backbone vector is devoid of the left-hand ITR, the packaging signal and E1 sequences.

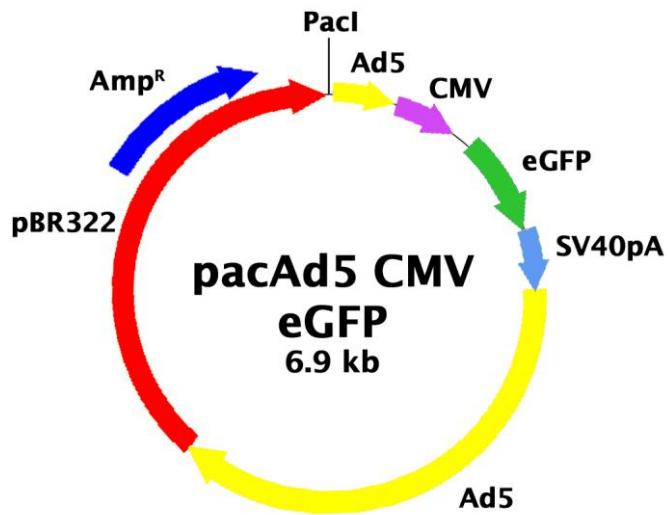


Figure 3. pacAd5 CMV-GFP Control Vector (6935 bp, Ampicillin-resistant).

pacAd5 CMV-GFP Features:

3-10:	PacI
16-368:	1-353 of Ad5
385-912:	CMV Promoter
992-1711:	GFP
1713-2160:	SV40 pA
2161-4615:	3328-5792 of Ad5
5867-6727:	β Lactamase

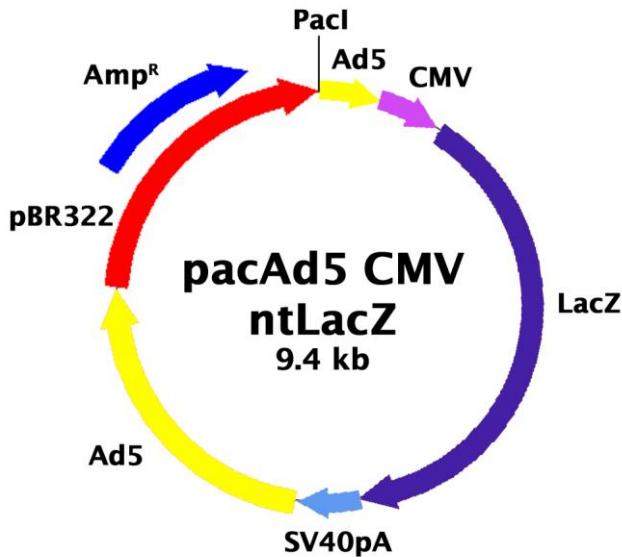


Figure 4. pacAd5 CMV-ntLacZ Control Vector (9278 bp, Ampicillin-resistant).

pacAd5 CMV-ntLacZ Features:

3-10:	PacI
16-368:	1-353 of Ad5
385-912:	CMV Promoter
1105-4148:	ntLacZ
4193-4640:	SV40 pA
4641-7095:	3328-5792 of Ad5
8347-9210:	β Lactamase

Preparation of Recombinant Adenovirus

I. Vector Linearization with PacI

1. Digest a sufficient amount of the pacAd5 CMV-IRES-GFP shuttle vector containing gene of interest and the pacAd5 9.2-100 Ad backbone vector with PacI.
2. Run 0.5 μ g of each digested DNA and undigested DNA on a 0.8% agarose gel to confirm the completion of PacI digestion (For pacAd5 9.2-100, one band of ~33 kb and a second band of 2.0 kb).
3. Remove buffer and enzyme from the remainder of the restriction reactions by phenol extraction/ethanol precipitation or using a similar DNA purification kit.
4. Resuspend the DNA in sterile dH₂O. Store the digested DNA at -20°C.

II. Transfection

1. Seed 2 \times 10⁶ cells in a 60 mm culture dish without antibiotics one day before transfection.

2. After 16 to 24 hours, start transfection when the culture becomes 70-80% confluence.
Note: We suggest transfecting cells with FuGENE® Transfection Reagent (Roche Applied Science) or Lipofectamine™ Plus (Invitrogen). For example, 4 µg of pacAd5 CMVK-NpA shuttle vector and 1 µg of pacAd5 9.2-100 Ad backbone vector are mixed with 9 µL FuGENE® Transfection Reagent according to the manufacturer's recommendation. The mixed DNA-FuGENE® complex is added by dropwise into the culture media.
3. Aspirate the media containing transfection reagent the next day and add 4 mL of complete culture medium.
4. After incubating for 7 days, check for the presence of plaques. If plate is ready for harvest, (>50% of cells lifted), then collect the Crude Viral Lysate. If not, feed the cells with 1 mL of complete culture medium, continue to incubate at 37°C with CO₂.
5. On day 10, check for the presence of plaques. If plate is ready for harvest, (>50% of cells lifted), then collect the Crude Viral Lysate. If not, feed the cells with 1 mL of complete culture medium, continue to incubate at 37°C with CO₂. Keep checking plate for the presence of plaques. Do not keep plate more than 15 days.

III. Harvesting the Crude Viral Lysate

1. Harvest adenovirus-containing cells by squirting cells off the plate with a 5 or 10 mL sterile serological pipette. Transfer cells and media to a sterile 15 ml tube. Scrape the cells into the medium with a cell lifter if necessary.
2. Release viruses from cells by three freeze/thaw cycles (10 minutes each in 37°C water bath and dry ice-methanol bath).
3. Centrifuge the cell lysate in a table-top centrifuge at 3000 rpm for 15 minutes at room temperature to pellet the cell debris.
4. Aliquot and store the Crude Viral Lysate (Initial Viral Stock) at -80°C.

IV. Amplification

Note: The following procedure is suggested for T75 flasks and may be optimized to suit individual needs.

1. Seed 3-5 x 10⁶ cells in a T75 flask one day before infection.
2. Add 50% of the above Crude Viral Lysate to the culture. We recommend using a multiplicity of ≥ 0.5 PFU (plaque forming units) or enough viruses that cells demonstrate cytopathic effects (CPEs) within 48 hrs.
3. During 24 - 48 hr infection, examine the monolayer twice per day under the microscope for CPE. When CPE is nearly complete (i.e. most cells rounded but not yet detached from the flask), harvest cells by pipetting media up and down to wash the infected cells from the flask into the media.
4. Pool infected cells and medium. Pellet cells by centrifugation at 1000 g for 5 minutes. Remove supernatant, resuspend cell pellet in medium or in 10 mM Tris, pH 8.0, 100 mM NaCl. (0.25-0.5 mL per T75 flask).
5. Release the adenoviruses from the cell suspension with three freeze/thaw cycles. Centrifuge at 3000 g for 10 minutes to pellet the cell debris. Discard the pellet and save supernatant as viral stock.
6. The viral supernatant can be stored at -80°C or immediately purified or titered.

Example of Results

The following figures demonstrate typical results of generating recombinant adenovirus. One should use the data below for reference only. This data should not be used to interpret actual results.

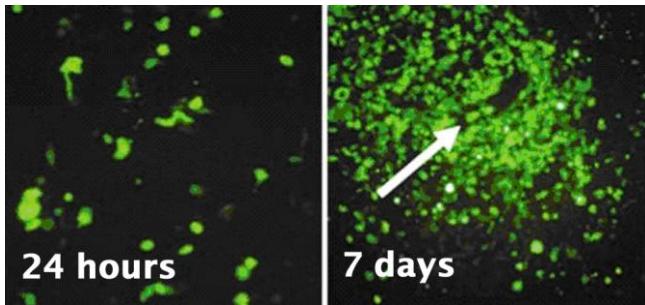


Figure 5. Generation of recombinant adenovirus using the RAPAd® Adenoviral Expression System. 293 cells were transfected with PacI linearized pacAd5 CMV-GFP vector and pacAd5 9.2-100 vector. Plates were examined for the presence of viral foci under inverted fluorescence microscope.

Appendix

pacAd5 CMV-IRES-GFP Plasmid Sequence

TAAGCTGGGATGGGTGCATACGTGGGATATGAGATGCATCTGGACTGTATTTAGTTGGCTATGTTCCCAGCCATATCCCCTGGGGATTCATGTTGTG
 CAGAACCCACCAGCACAGTGTATCCGGTCACTGGAAATTGTCACTGTAAGCTTAGAAGGAAATGGCTGGAAGAACCTGGAGACGCCCTGTGACCTCAAGA
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 GCGCCCTTCTGCTTCAAGAA

References

1. Bett AJ, Haddara W, Prevec L and Graham FL. (1994) *Proc Natl Acad Sci U S A*. **91**:8802-6 (Homologous recombination in packaging cell line)
2. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W. et al. (1998) *Proc Natl Acad Sci U S A* **95**:2509-14. (pAdEasy System)
3. R D Anderson, R E Haskell, H Xia, B J Roessler and B L Davidson (2000) *Gene Ther.* **7**:1034-8. (RAPAd® System).

Recent Product Citations

1. Ložić, M. et al. (2015). Overexpression of oxytocin receptors in the hypothalamic PVN increases baroreceptor reflex sensitivity and buffers BP variability in conscious rats. *Br J Pharmacol.* **171**:4385-4398.
2. Brunton, P. J. et al. (2015). 5 α -reduced neurosteroids sex-dependently reverse central prenatal programming of neuroendocrine stress responses in rats. *J Neurosci.* **35**:666-677.
3. Ložić, M. et al. (2014). Overexpression of oxytocin receptors in the hypothalamic PVN increases baroreceptor reflex sensitivity and buffers BP variability in conscious rats. *Br J Pharmacol.* **171**:4385-4398.

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